ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1995, p. 790–792 Vol. 39, No. 3
0066-4804/95/$04.00+0

nfxC-Type Quinolone Resistance in a Clinical Isolate of *Pseudomonas aeruginosa*

HIDEYUKI FUKUDA,[1]* MASAKI HOSAKA,[1] SHIZUKO IYOBE,[2] NAOMASA GOTOH,[3] TAKESHI NISHINO,[3] AND KEIJI HIRAI[1]

Central Research Laboratories, Kyorin Pharmaceutical Co., Ltd., Nogi-machi, Shimotsuga-gun, Tochigi-ken,[1] *Laboratory of Drug Resistance in Bacteria, School of Medicine, Gunma University, Maebashi, Gunma-ken,*[2] *and Kyoto Pharmaceutical University, Misasaginakauchi, Yamashina, Kyoto-fu,*[3] *Japan*

Received 27 June 1994/Returned for modification 3 October 1994/Accepted 9 January 1995

Quinolone resistance gene *nqr-T91* in a clinical isolate of *Pseudomonas aeruginosa* P1481 was cotransducible with *catA1* in *P. aeruginosa* PAO. The *nqr-T91* transductant, PKH-T91, was resistant to norfloxacin, imipenem, and chloramphenicol and showed less norfloxacin accumulation than the parent strain did. Loss of the 46-kDa outer membrane protein (D2) and an increase in the 50-kDa outer membrane protein in PKH-T91 were observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lipopolysaccharides in the transductant were also changed. These alterations were considered to be related to lower levels of norfloxacin accumulation in PKH-T91. These genetic and biochemical properties suggested that an *nfxC* type of quinolone-resistant mutation occurred in a clinical isolate of *P. aeruginosa* P1481.

New quinolones have potent in vitro antimicrobial activities and are therapeutically effective against gram-positive and gram-negative bacterial infections, including *Pseudomonas aeruginosa* infection. Recently, quinolone-resistant clinical isolates of *P. aeruginosa* have emerged because of greater and wider clinical usage of new quinolones (1, 9, 18, 31).

Quinolone resistance in bacteria can be attributed to two mechanisms, (i) alteration of DNA gyrase and (ii) alteration of membrane permeability. These mechanisms have also been found in *P. aeruginosa*. Four chromosomal mutations, *nfxA* (*nalA*), *nfxB*, *nfxC*, and *nalB* (*cfxB*), that confer quinolone resistance have been identified and mapped on the *P. aeruginosa* PAO chromosome (10, 14, 28, 30). The *nfxA* and *nalA* genes are alleles of *gyrA* that encode DNA gyrase subunit A, while the other genes are associated with the membrane permeability of new quinolones. In clinical isolates of *P. aeruginosa*, alteration of DNA gyrase or membrane permeability has been reported to be one of the quinolone resistance mechanisms (6, 8, 17, 22, 25, 29, 34).

We previously reported on the mechanism of quinolone resistance in a clinical strain of *P. aeruginosa* P1481 (2) that had been isolated from the urine of a patient treated with norfloxacin in a Japanese hospital and had shown high-level quinolone resistance. The quinolone resistance gene of P1481, *nqr-T81*, has been reported to be cotransducible with *eda* (*hex*)-*9001*, which is known to be cotransducible with *nfxA* (*gyrA*), on the *P. aeruginosa* PAO chromosome (2). DNA gyrase subunit A purified from P1481 showed resistance to quinolones (2). Thus, quinolone resistance in P1481 was associated with an alteration of DNA gyrase. However, P1481 showed less susceptibility to new quinolones than the *gyrA* mutant did (2). It was difficult to attribute the high level of quinolone resistance in this strain only to alteration of DNA gyrase. In this study, we examined the quinolone resistance mechanisms in P1481 in addition to alteration of DNA gyrase (*gyrA* mutation).

Table 1 shows the antimicrobial susceptibilities of P1481 and the other strains used in this study. Antimicrobial susceptibility (MIC) was measured by an agar dilution method with Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) (13). Ciprofloxacin, fleroxacin, norfloxacin, and ofloxacin were synthesized at the Central Research Laboratories of Kyorin Pharmaceutical Co., Ltd. Chloramphenicol and gentamicin were purchased from Sigma Chemical Co., St. Louis, Mo. Carbenicillin and imipenem were purchased from Fujisawa Pharmaceutical Co., Ltd., and Banyu Pharmaceutical Co., Ltd., Tokyo, Japan, respectively.

Quinolone-resistant strain P1481 also showed cross-resistance to imipenem and chloramphenicol. Plasmid pPAW207, which carried the wild-type *Escherichia coli gyrA* gene (34), was introduced into P1481 by a transformation technique described previously (21). With the introduction of pPAW207, P1481 partially lost its resistance to norfloxacin; that is, the MICs of norfloxacin against P1481 and its transformant were 50 and 12.5 μg/ml, respectively. This indicated that the *gyrA* mutation in P1481 was restored by pPAW207. However, the transformant of P1481 was still resistant to norfloxacin compared with PAO 4032, a quinolone-susceptible strain of *P. aeruginosa* (MIC of norfloxacin, 0.39 μg/ml). These findings indicated that a quinolone resistance mutation in addition to the one in *gyrA* occurred in P1481.

We previously reported that an *nfxC* quinolone-resistant mutant of *P. aeruginosa* PAO also showed cross-resistance to imipenem and chloramphenicol and that the *nfxC* gene was cotransducible with *catA1* (10). Thus, transduction analysis of a quinolone resistance gene other than *gyrA* in P1481 was performed. Transduction with phage G101 was carried out by the method of Matsumoto et al. (24). *P. aeruginosa* PAO 4032 (*met-9020 catA1* [23] *nar-9011 mtu-9002 tyu-9030 dcu-9013*) was used as the recipient strain. Ninety-four *catA1*$^+$ transductants were obtained. All of these transductants were resistant to norfloxacin. The quinolone resistance gene in P1481, *nqr-T91*, was cotransducible with *catA1* (100% linkage). Additional control experiments indicated that the acquisition of *catA*$^+$ itself was not responsible for conferring the NfxC resistance phenotype. Table 1 shows the antimicrobial susceptibilities of the representative transductant, designated PKH-T91. The susceptibilities of PKH-T91 to new quinolones were 16- to

* Corresponding author. Mailing address: Central Research Laboratories, Kyorin Pharmaceutical Co., Ltd., 2399-1, Mitarai, Nogi-machi, Shimotsuga-gun, Tochigi-ken, 329-01, Japan. Phone: 0280 (56) 2201. Fax: 0280 (57) 1293.

TABLE 1. Antimicrobial susceptibilities of the strains used in this study

Strain	Antimicrobial agent	MIC (μg/ml)
P1481	Norfloxacin	50
	Fleroxacin	50
	Ofloxacin	50
	Ciprofloxacin	12.5
	Carbenicillin	12.5
	Imipenem	6.25
	Chloramphenicol	>200
	Gentamicin	3.13
P1481(pPAW207)	Norfloxacin	12.5
	Fleroxacin	12.5
	Ofloxacin	12.5
	Ciprofloxacin	3.13
	Carbenicillin	>100
	Imipenem	6.25
	Chloramphenicol	>200
	Gentamicin	3.13
PAO 4032	Norfloxacin	0.39
	Fleroxacin	0.39
	Ofloxacin	0.78
	Ciprofloxacin	0.10
	Carbenicillin	50
	Imipenem	0.78
	Chloramphenicol	25
	Gentamicin	6.25
PKH-T91	Norfloxacin	6.25
	Fleroxacin	12.5
	Ofloxacin	6.25
	Ciprofloxacin	1.56
	Carbenicillin	50
	Imipenem	6.25
	Chloramphenicol	>200
	Gentamicin	6.25

32-fold lower than those of the parent strain, PAO 4032. PKH-T91 was also cross-resistant to imipenem and chloramphenicol as the *nfxC* mutant of *P. aeruginosa* PAO. These findings suggested that the *nfxC*-type quinolone-resistant mutation might occur in P1481.

The *nfxC* mutation in *P. aeruginosa* PAO was an impermeability-type quinolone-resistant mutation (10). The accumulation of norfloxacin in bacterial cells was studied after exposure to 10 μg of norfloxacin per ml for 20 min at 37°C as described previously (14). The accumulations of norfloxacin were 307 and 141 ng/mg of dry cells of PAO 4032 and PKH-T91, respectively. PKH-T91 showed about a twofold reduction in the accumulation of norfloxacin compared with that of PAO 4032. This *nfxC*-type norfloxacin resistance in P1481 was caused by lower accumulation of this drug.

Quinolone accumulation in bacterial cells is defined by two factors, influx and efflux of the drug. Recently, the quinolone efflux systems in some bacteria were studied (16, 33). Thus, norfloxacin accumulation was measured in the presence of 250 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) as described previously (4). By treatment with CCCP 5 min before exposure to norfloxacin, the accumulations in PAO 4032 and PKH-T91 increased to 350 and 281 ng/mg of dry cells, respectively. The accumulation in PAO 4032 was slightly increased, and the accumulation in PKH-T91 was about twofold higher than that in the absence of CCCP. The accumulation of norfloxacin in PKH-T91 was more affected by treatment with CCCP than that in PAO 4032. These findings suggest that an energy-dependent quinolone efflux system might contribute to

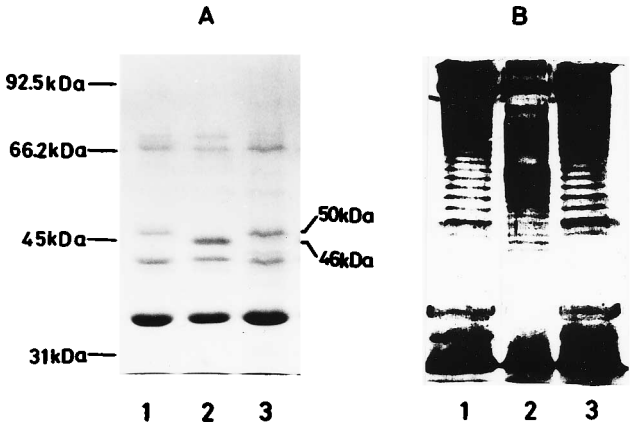

FIG. 1. Outer membrane fraction profiles of P1481, PAO 4032, and PKH-T91, the *nqr-T91* transductant. (A) OMP profiles. Lanes: 1, P1481 (a quinolone-resistant clinical isolate); 2, PAO 4032 (a quinolone-susceptible strain); 3, PKH-T91 (a quinolone-resistant *nqr-T91* transductant). Phospholipase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa) were used as molecular mass standards. (B) LPS profiles. Lanes: 1, P1481; 2, PAO 4032; 3, PKH-T91.

lower norfloxacin accumulation in PKH-T91. However, the accumulation of norfloxacin in PKH-T91 in the presence of CCCP was still lower than that in PAO 4032. This suggests that quinolone influx is also associated with lower norfloxacin accumulation in PKH-T91. In PKH-T91, reduced norfloxacin accumulation might result from the interaction of influx and active efflux, as described for a *marR* mutant of *E. coli* (7).

In quinolone-resistant *P. aeruginosa* strains, alterations of outer membrane components (outer membrane proteins [OMPs] and lipopolysaccharides [LPSs]) have been reported (4–6, 8, 10, 12, 14, 20, 22, 25, 26). We have also reported a decrease for the 46-kDa OMP, D2 protein, and an increase for the 50-kDa OMP in an *nfxC* mutant of *P. aeruginosa* PAO (10). Therefore, outer membrane fractions of P1481, PAO 4032, and PKH-T91, its *nqr-T91* transductant, were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11, 15, 19). OMPs were prepared by the methods of Poxton et al. (27). Figure 1A shows these OMP profiles. In P1481, the 46-kDa OMP was not detected, but large amounts of the 50-kDa OMP appeared. The same changes in OMPs were observed in PKH-T91 as in the parent strain. The OMP profiles revealed the occurrence of *nfxC*-like alterations in strains P1481 and PKH-T91. LPSs were prepared by the method of Hitchcock and Brown (15). Figure 1B shows the ladder patterns of LPSs. There were changes in the ladder patterns of LPSs between quinolone-resistant strains and the quinolone-susceptible strain. This suggests that changes in OMPs and/or LPSs might be associated with lower accumulations of norfloxacin in quinolone-resistant strains.

The 46-kDa OMP (D2 protein) is known to play an important role in the outer membrane permeability of carbapenems, including imipenem (32). Loss of the 46-kDa OMP might correlate with the decrease in imipenem permeation across the outer membranes of P1481 and PKH-T91. Michéa-Hamzehpour et al. indicated that quinolone-resistant *P. aeruginosa* strains with cross-resistance to imipenem had less D2 protein and hypothesized that fluoroquinolones also permeated through D2 protein in *P. aeruginosa* (25, 26). We have obtained spontaneous imipenem-resistant mutants of *P. aeruginosa* PAO. Although these strains had no D2 OMP, they showed no cross-resistance to new quinolones (data not shown). Further-

more, some reports suggested that an increase or appearance of specific OMPs played an important role in the decrease of quinolone accumulation in quinolone-resistant strains of *P. aeruginosa* (4, 10, 12, 14). Therefore, norfloxacin permeation across the outer membrane was considered to be little affected by loss of the 46-kDa OMP alone. Thus, quinolone accumulation in P1481 and PKH-T91 might be affected by increases in the 50-kDa OMP and/or changes in LPSs.

We have shown that the *nfxB*-type quinolone resistance mechanism exists in clinical isolates of *P. aeruginosa* (17). Aubert et al. reported the development of a possible NfxC phenotype (resistance to quinolone and imipenem) in a clinical isolate of *P. aeruginosa* from a patient after treatment with quinolone only (3). However, we provide for the first time both genetic and biochemical lines of evidence for the occurrence of *nfxC*-type quinolone resistance mutations in clinical isolates of *P. aeruginosa*. Alterations of both DNA gyrase (2) and membrane permeability were related to the high level of quinolone resistance in a clinical isolate of *P. aeruginosa* P1481.

We thank H. Matsumoto for providing *P. aeruginosa* PAO and H. Yoshida for providing plasmid pPAW207.

REFERENCES

1. **Acar, J. F., and S. Francoual.** 1990. The clinical problems of bacterial resistance to the new quinolones. J. Antimicrob. Chemother. **26**(Suppl. B)**:** 207–213.
2. **Aoyama, H., K. Hirai, T. Irikura, S. Iyobe, and S. Mitsuhashi.** 1989. New quinolone resistance in clinical isolates of *Pseudomonas aeruginosa*. Rev. Infect. Dis. **2**(Suppl. 5)**:**S972–S973.
3. **Aubert, G., B. Pozzetto, and G. Dorche.** 1992. Emergence of quinolone-imipenem cross-resistance in *Pseudomonas aeruginosa* after fluoroquinolone therapy. J. Antimicrob. Chemother. **29:**307–312.
4. **Celesk, R. A., and N. J. Robillard.** 1989. Factors influencing the accumulation of ciprofloxacin in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **33:**1921–1926.
5. **Chamberland, S., A. S. Bayer, T. Schollaardt, S. A. Wong, and L. E. Bryan.** 1989. Characterization of mechanisms of quinolone resistance in *Pseudomonas aeruginosa* strains isolated in vitro and in vivo during experimental endocarditis. Antimicrob. Agents Chemother. **33:**624–634.
6. **Chin, N. X., N. Clynes, and H. C. Neu.** 1989. Resistance to ciprofloxacin appearing during therapy. Am. J. Med. **87**(Suppl. 5A)**:**28S–31S.
7. **Cohen, S. P., L. M. McMurry, D. C. Hooper, J. S. Wolfson, and S. B. Levy.** 1989. Cross-resistance to fluoroquinolones in multiple-antibiotic-resistant (Mar) *Escherichia coli* selected by tetracycline or chloramphenicol: decreased drug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrob. Agents Chemother. **33:**1318–1325.
8. **Diver, J. M., T. Schollaardt, H. R. Rabin, C. Thorson, and L. E. Bryan.** 1991. Persistence mechanisms in *Pseudomonas aeruginosa* from cystic fibrosis patients undergoing ciprofloxacin therapy. Antimicrob. Agents Chemother. **35:**1538–1546.
9. **Eron, L. J., L. Harvey, D. L. Hixon, and D. M. Poretz.** 1985. Ciprofloxacin therapy of infections caused by *Pseudomonas aeruginosa* and other resistant bacteria. Antimicrob. Agents Chemother. **28:**308–310.
10. **Fukuda, H., M. Hosaka, K. Hirai, and S. Iyobe.** 1990. New norfloxacin resistance gene in *Pseudomonas aeruginosa* PAO. Antimicrob. Agents Chemother. **34:**1757–1761.
11. **Hancock, R. E. W., and H. Nikaido.** 1978. Outer membranes of gram-negative bacteria. XIX. Isolation from *Pseudomonas aeruginosa* PAO1 and use in reconstitution and definition of the permeability barrier. J. Bacteriol. **136:**381–390.
12. **Hirai, K.** 1989. Quinolone-resistance mechanism involved in outer membrane, p. 187–201. *In* P. B. Fernandes (ed.), International telesymposium on quinolones. J. R. Prous Science Publishers, Barcelona, Spain.
13. **Hirai, K., H. Aoyama, S. Suzue, T. Irikura, S. Iyobe, and S. Mitsuhashi.** 1986. Isolation and characterization of norfloxacin-resistant mutants of *Escherichia coli* K-12. Antimicrob. Agents Chemother. **30:**248–253.
14. **Hirai, K., S. Suzue, T. Irikura, S. Iyobe, and S. Mitsuhashi.** 1987. Mutations producing resistance to norfloxacin in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **31:**582–586.
15. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
16. **Hooper, D. C., J. S. Wolfson, K. S. Souza, E. Y. Ng, G. L. McHugh, and M. N. Swartz.** 1989. Mechanisms of quinolone resistance in *Escherichia coli*: characterization of *nfxB* and *cfxB*, two mutant resistance loci decreasing norfloxacin accumulation. Antimicrob. Agents Chemother. **33:**283–290.
17. **Jakics, E. B., S. Iyobe, K. Hirai, H. Fukuda, and H. Hashimoto.** 1992. Occurrence of the *nfxB* type mutation in clinical isolates of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36:**2562–2565.
18. **Kresken, M., D. Hafner, H. Mittermayer, L. Verbist, E. Berezin, H. Giamarellou, S. Esposito, B. van Klingere, F. K. Kayser, D. S. Reeves, B. Wiedemann, and Study Group.** 1994. Prevalence of fluoroquinolone resistance in Europe. Infection **22**(Suppl. 2)**:**S90–S98.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
20. **Legakis, N. J., L. S. Tzouvelekis, A. Marikis, and H. Kotsifaki.** 1989. Outer membrane alterations in multiresistant mutants of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **33:**124–127.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Masecar, B. L., R. A. Celesk, and N. J. Robillard.** 1990. Analysis of acquired ciprofloxacin resistance in a clinical strain of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **34:**281–286.
23. **Matsumoto, H., T. Nakazawa, S. Ohota, and Y. Terawaki.** 1981. Chromosomal locations of *catA*, *pobB*, *pcaA*, *dcu* and *chu* genes in *Pseudomonas aeruginosa*. Genet. Res. **38:**251–266.
24. **Matsumoto, H., S. Ohta, R. Kobayashi, and Y. Terawaki.** 1978. Chromosomal location of genes participating in the degradation purines in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **167:**165–176.
25. **Michéa-Hamzehpour, M., Y. X. Furet, and J.-C. Pechère.** 1991. Role of protein D2 and lipopolysaccharide in diffusion of quinolones through the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **35:**2091–2097.
26. **Michea-Hamzehpour, M., C. Lucain, and J.-C. Pechere.** 1991. Resistance to pefloxacin in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **35:** 512–518.
27. **Poxton, I. R., G. T. Bell, and G. R. Barclay.** 1985. The association on SDS-polyacrylamide gels of lipopolysaccharide and outer membrane proteins of *Pseudomonas aeruginosa* as revealed by monoclonal antibodies and western blotting. FEMS Microbiol. Lett. **79:**31–36.
28. **Rella, M., and D. Haas.** 1982. Resistance of *Pseudomonas aeruginosa* PAO to nalidixic acid and low levels of β-lactam antibiotics: mapping of chromosomal genes. Antimicrob. Agents Chemother. **22:**242–249.
29. **Robillard, N. J.** 1990. Broad-host-range gyrase A gene probe. Antimicrob. Agents Chemother. **34:**1889–1894.
30. **Robillard, N. J., and A. L. Scarpa.** 1988. Genetic and physiological characterization of ciprofloxacin resistance in *Pseudomonas aeruginosa* PAO. Antimicrob. Agents Chemother. **32:**535–539.
31. **Scully, B. F., H. C. Neu, M. F. Parry, and W. Mandell.** 1986. Oral ciprofloxacin therapy of infections due to *Pseudomonas aeruginosa*. Lancet **i:**819–822.
32. **Trias, J., and H. Nikaido.** 1990. Outer membrane protein D2 catalyzes facilitated diffusion of carbapenems and penems through the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **34:**52–57.
33. **Yoshida, H., M. Bogaki, S. Nakamura, K. Ubukata, and M. Konno.** 1990. Nucleotide sequence and characterization of the *Staphylococcus aureus norA* gene, which confers resistance to quinolones. J. Bacteriol. **172:**6942–6949.
34. **Yoshida, H., M. Nakamura, M. Bogaki, and S. Nakamura.** 1990. Proportion of DNA gyrase mutants among quinolone-resistant strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **34:**1273–1275.